Procedure & Checklist – No-Amp Targeted Sequencing Utilizing the CRISPR-Cas9 System

This procedure describes sequencing targeted genomic regions without amplification on the PacBio® Sequel®, Sequel II, and Sequel IIe Systems (Sequel Systems) by using a CRISPR-Cas9 enrichment methodology. Genomic DNA (gDNA) is first treated to prevent any termini arising during fragmentation from participating in SMRTbell® library preparation. The treated gDNA is subsequently subjected to targeted CRISPR-Cas9 digestion.

Cas9 nuclease, in association with two individual guide RNA (gRNA) oligonucleotides, identifies and then cleaves a specific recognition site on each side of the target region. Different regions of interest can be simultaneously targeted in a single CRISPR-Cas9 digest reaction by using multiple gRNA pairs. After RNA-directed cleavage, adapters are ligated to the blunt template ends to form SMRTbell templates. In the final step, unligated DNA is eliminated by nuclease digestion.

Note that this procedure is to be used in conjunction with customer-designed assays. Assay design involves generating and selecting CRISPR-Cas9 RNA oligonucleotides that will excise a specific target region of interest. CRISPR-Cas9 RNA oligos are ordered through a third-party vendor.

This document presents an example No-Amp targeted sequencing workflow that has been tested and validated for analyzing the following human genomic regions:

1) CAG repeat locus at the 5' end of the *HTT* gene (4p16.3)
2) CGG repeat locus in the 5' UTR of the *FMR1* gene (Xq27.3)
3) ATTCT repeat locus in intron 9 of the *ATXN10* gene (22q13.31)
4) Intronic GGGGCC repeat locus of the *C9orf72* gene (9p21.2)
5) 14 Ataxia-related repeat elements

Ordering information for RNA oligos specific to these targets is listed in Appendix A. Technical guidance on design of CRISPR-Cas9 RNA oligonucleotides for other targets is provided in *Reference Guide – Designing CRISPR-Cas9 RNA Oligonucleotides for the No-Amp Targeted Sequencing Procedure*.

DNA Input Requirements

The amount of gDNA required per experiment depends on the number of samples to be multiplexed and are summarized in Table 1 below.

# of Samples	Minimum Input gDNA per Sample (ng)*	Maximum Input gDNA per Sample (ng)**
1	5000	24000
2	2500	12000
3	1700	8000
4	1250	6000
5	1000	4800
10	500	2400
11	500	2182
48	500	500

* When multiplexing ≥10 samples, Minimum Input gDNA per sample (ng) is 500 ng
** Maximum Input gDNA per sample (ng)** = 24000 ng / # of samples

Multiplexing up to 48 samples is supported with a minimum starting input of 0.5 µg gDNA per sample. We recommend measuring the amount of double-stranded DNA using a fluorometric quantitation method such as the Qubit® Quantitation Platform.

Evaluate gDNA Quality

Optimal results are achieved with high-purity, high-molecular weight gDNA. Before starting this procedure, we recommend evaluating gDNA size and integrity using a pulsed-field electrophoresis system. Since the workflow performance depends on gDNA integrity, a system that provides good separation and resolution of high-molecular weight DNA is necessary. Any of the three commercially available systems listed in Table 2 below may be used to evaluate gDNA quality.

Method	Procedure/Product Note
Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper® XA Pulsed Field Electrophoresis System
Sage Science™ Pippin Pulse Electrophoresis Power Supply	Procedure & Checklist - Using the Sage Science™ Pippin Pulse Electrophoresis Power Supply System
Agilent FEMTO Pulse™ System	Product Note - Fast, High-Resolution DNA Sizing with the Agilent Femto Pulse System

Table 2. Genomic DNA quality evaluation methods.

For best results, ensure that the fragment size mode for your starting gDNA sample is ≥50 kilobase pairs (see Figure 1 below). For gDNA samples with a fragment size mode <50 kb, increasing the gDNA input amount for a single sample above 10 µg can help increase the yield of on-target reads.

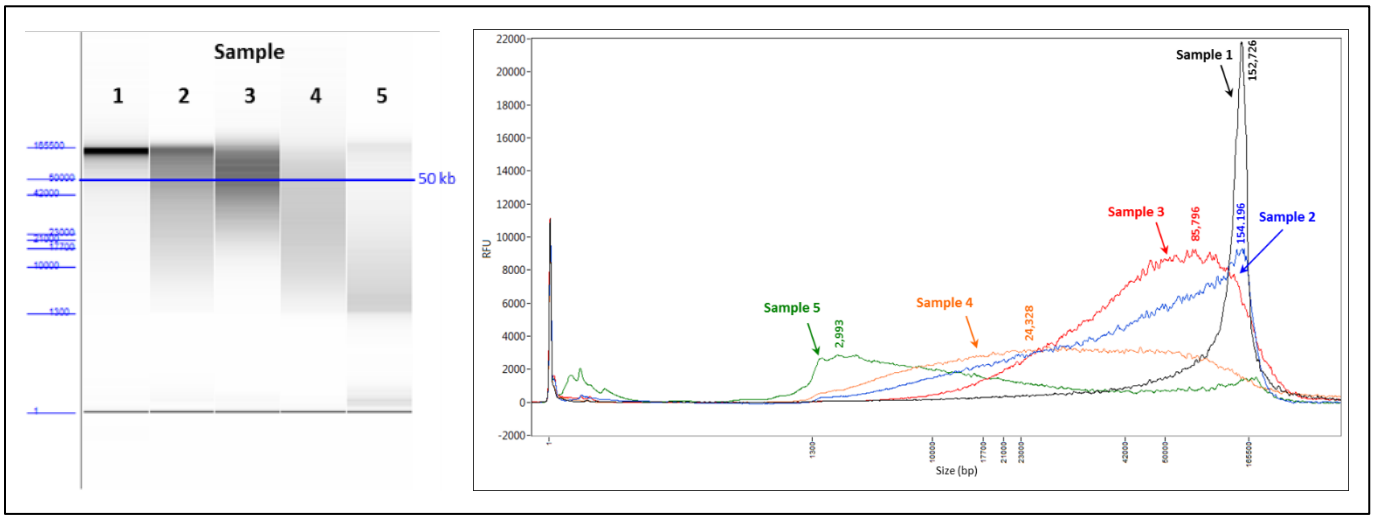


Figure 1. Evaluation of gDNA quality using a Femto Pulse System. Samples 1 through 5 depict a wide degree of DNA integrity with various amounts of degradation. The majority of fragments for Samples 1, 2 and 3 are above 50 kb while those for Sample 4 and 5 are below 50 kb. While using increased gDNA input amounts may generate levels of on-target reads for Sample 4 comparable to Samples 1, 2 and 3, the level of degradation for Sample 5 is such that very few intact target molecules may be obtained with the maximum amount of gDNA input of 24 µg.

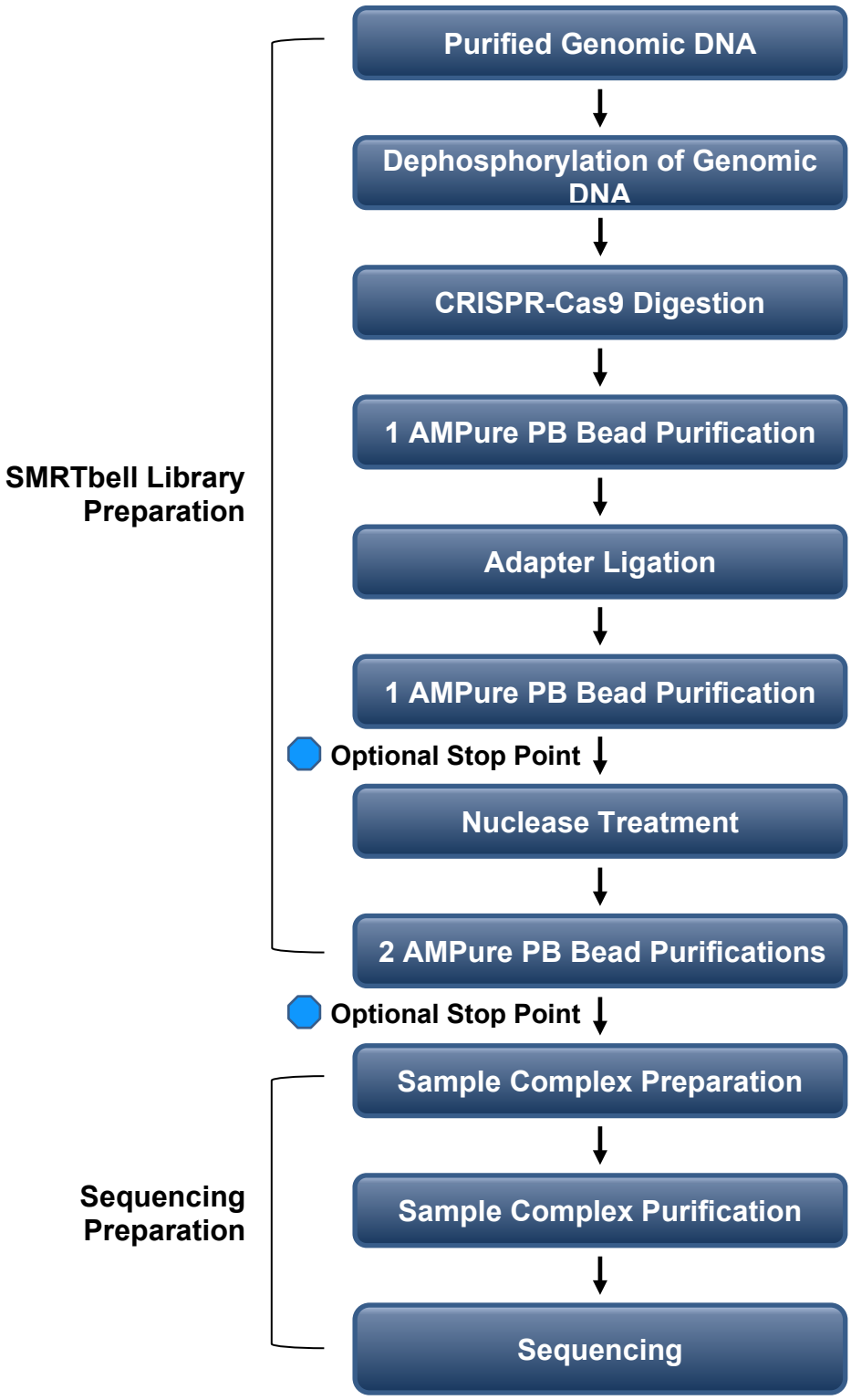
Assessment of gDNA purity can be achieved by examining the 260 nm to 280 nm ($OD_{260/280}$) and 260 nm to 230 nm ($OD_{260/230}$) absorbance ratios obtained by spectrophotometric analysis. Genomic DNA should have $OD_{260/280}$ and $OD_{260/230}$ ratios of 1.8-2.0 and 2.0-2.2, respectively. Superior performance may be achieved by re-purifying gDNA samples with AMPure® PB beads if $OD_{260/280}$ and $OD_{260/230}$ ratios fall substantially outside the ideal value ranges.

For cases where the yield of on-target reads obtained is lower than expected, there may have been contaminants carried over and affecting overall sequencing performance. In such cases, additional purification of the gDNA sample may be necessary. If needed, we recommend using the Qiagen DNeasy PowerClean Pro Kit to remove carry-over contaminants in gDNA samples to be used for the No-Amp procedure; however, note that post-cleanup recovery can be as low as 20% depending on the sample. This should be taken into account when planning the purification steps to ensure that you have sufficient gDNA for your No-Amp experiment.

Best Practices for Generating No-Amp Targeted Sequencing Results

1. Use DNA extraction methods optimized for the gDNA source material.
2. Use the required DNA input amount specified in the DNA Input Requirements section.
3. Prepare genomic DNA samples for use by equilibrating to room temperature and mixing the samples thoroughly by gently inverting the tube several times prior to start of the procedure.
4. Viscous genomic DNA samples should be diluted, if concentration allows, to ensure accurate quantitation prior to start of the procedure and efficient processing in the procedure.
5. Maintain enzymes at -20°C in a benchtop cooler during reaction setup. Return enzymes to storage once reaction setup is complete.
6. Mix reactions as instructed. Reaction preparations from the gDNA Treatment step to Adapter Ligation step contain high-molecular weight gDNA that can become fragmented from improper handling during reaction setup. **Any fragment ends generated after gDNA treatment will likely increase the amount of off-target SMRTbell templates in the final SMRTbell library.**
7. Use a thermocycler or microcentrifuge tube incubator equipped with heated lid for all incubation steps involving enzymatic reactions to minimize water evaporation and condensation. If the instrument allows, set the lid temperature 10 °C above the incubation temperature.
8. Use dedicated nuclease-free water and consumables when working with RNA oligos. Keep RNA oligos and associated reagents for CRISPR-Cas9 digestion on ice at all times.
9. Allow sufficient time for AMPure PB beads and Elution Buffer to reach room temperature before use.
10. When performing sequential AMPure PB bead purifications, transferring a small number of beads from the first purification into the binding step of the second purification is preferred over leaving some eluate behind to minimize sample loss.
11. Slowly remove supernatant and ethanol washes during AMPure PB bead purifications to minimize bead loss due to fluid motion.
12. Be mindful not to over-dry AMPure PB beads during purification steps.
13. We recommend including a validated target locus (e.g., the HTT target using the recommended guide RNAs in this procedure) as an internal control for your No-Amp experiment.

No-Amp Library Construction Workflow



Required Materials

Third-Party Equipment, Consumables and Reagents

Item	Vendor	Part Number	Comments
0.5 mL and 1.5 mL DNA LoBind Tubes	Eppendorf	0030108035 and 0030108051	
0.2 mL PCR Thermal Cycling Tubes	USA Scientific	various	
Molecular Biology Pipettes, Standard Set	MLS	-	capable of pipetting volumes 0.5 µL to 1 mL
Benchtop Cooler	MLS	-	for maintaining enzymes at -20°C
Microcentrifuge	MLS	-	14,000 RCF capability
Mini Centrifuge	MLS	-	for quick spins
Vortex Mixer	MLS	-	2,000 rpm capability
DynaMag™-2 Magnet	Thermo Fisher Scientific	12321D	16 x 1.5-2.0 mL microcentrifuge tubes
MagneSphere® Technology Magnetic Separation Stand (optional)	Promega	Z5341	12 x 0.5 mL microcentrifuge tubes; optional magnetic stand
Neodymium Disc Magnet, 1/4" dia. by 1/8" thick (optional)	K&J Magnetics	D42-N52	used with stand Z5341 listed above
PCR Thermal Cycler	MLS	-	
ThermoMixer C with heated lid (or equivalent)	Eppendorf	5382000023	15-100ºC range
Qubit® Quantitation Platform (e.g.,Qubit 4 Fluorometer)	Thermo Fisher Scientific	Q33238	
Qubit™ 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230	
Nuclease-Free Water, not DPEC-Treated	Ambion	AM9937	10 x 50 mL
Nuclease-Free Duplex Buffer	IDT	11-01-03-01 or 11-05-01-12	10 x 2 mL or 300 mL
Ethyl Alcohol, Molecular Biology Grade	MLS	-	
Shrimp Alkaline Phosphatase (rSAP)	New England BioLabs	M0371S or M0371L	500 or 2,500 units @ 1,000 U/mL
CutSmart® Buffer, 10X	New England BioLabs	B7204S	5 mL each
Exonuclease III	New England BioLabs	M0206S or M0206L	5,000 or 25,000 units @ 100,000 U/mL
Cas9 Nuclease, *S. pyogenes*	New England BioLabs	M0386T or M0386M	400 or 2,000 pmol @ 20 µM
NEBuffer™ 3.1, 10X[1]	New England BioLabs	B7203S	5 mL each
T4 DNA Ligase Reaction Buffer, 10X	New England BioLabs	B0202S	6 mL each
T4 DNA Ligase, HC	Thermo Fisher Scientific	EL0013	5000 Weiss units @ 30 U/µL
SOLu-Trypsin	Sigma-Aldrich	EMS0004	100 µg @ 1 mg/mL
1 kb DNA Ladder (carrier DNA)	New England BioLabs	N3232S or N3232L	0.2 ml or 1 ml @ 500 µg/mL
Recombinant ribonuclease inhibitor	Takara Bio USA	2313A or 2313B	5000 or 25000 units @ 40U/µL

[1]NEBuffer™ 3.1, 10X is also included with NEB Cas9 Nuclease, *S. pyogenes*.

PacBio® Reagents and Consumables

Item	Vendor	Part Number	Comments
Pacific Biosciences® Binding Kits			
For Sequel® System: Sequel Binding and Internal Control Kit 3.0	PacBio	101-626-600	
For Sequel® II and IIe Systems: Sequel II Binding Kit 2.0 and Internal Control Kit 1.0	PacBio	101-842-900	
Pacific Biosciences® Sequencing Kits			
For Sequel® System: Sequel Sequencing Kit 3.0 (4 rxn)	PacBio	101-597-900 or	4-rxn kit
For Sequel® II and IIe Systems: Sequel II Sequencing Kit 2.0 (4 rxn)	PacBio	101-820-200	4-rxn kit
Pacific Biosciences® SMRT® Cells			
For Sequel® System: SMRT® Cell 1M v3 Tray (4 cells) or SMRT® Cell 1M v3 LR Tray (4 cells)	PacBio	101-531-000 or 101-531-001	10-hr max collection time or 20-hr max collection time
For Sequel II and IIe Systems: SMRT Cell 8M Tray (4 cells)	PacBio	101-389-001	
AMPure® PB	PacBio	100-265-900	1 bottle, 5 mL
Elution Buffer	PacBio	101-633-500	1 bottle, 50 mL
No-Amp Accessory Kit, containing:	PacBio	101-788-900	
– Sequencing Primer v4	PacBio	-	1 tube, 18 µL
– 10X Primer Buffer v2	PacBio	-	1 tube, 1000 µL
– 10X Annealing Buffer	PacBio	-	2 tubes, 1000 µL each
SMRTbell® Enzyme Clean up Kit	PacBio	101-746-400	

Required CRISPR-Cas9 RNA Oligonucleotides

In addition to the materials listed above, CRISPR-Cas9 RNA oligos that are compatible with customer-designed assays are required. Validated RNA oligos targeting HTT, FMR1, ATXN10, C9orf72 and Ataxia-related repeat elements in the human genome are listed in **Appendix A**.

You can also design your own RNA oligos for your specific target regions of interest. Please refer to *Reference Guide – Designing CRISPR-Cas9 RNA Oligonucleotides for the No-Amp Targeted Sequencing Procedure* for technical guidance on designing target-specific oligos. We recommend that you test several oligo designs before choosing a final crRNA design set for a particular target. .

Required Barcoded Adapters for Multiplexing

Barcoded adapters are required for multiplex sequencing. Up to 48 samples may be barcoded for multiplex sequencing on the Sequel II and IIe Systems. **Appendix B** contains a list of blunt-end Barcoded Adapter sequences that can be ordered from IDT or other third-party vendor. The **Oligo Order Sheet** and **FASTA file** for data analysis are available on our Multiplexing Page.

Barcoded oligos must be self-annealed to form SMRTbell adapter hairpins first before use. The procedure to self-anneal barcoded oligos is described in the next section below under “PacBio Barcoded Adapters for Multiplexing”. Note: Adapters require a 5’ phosphorylation modification And HPLC purification is recommended.

Initial Reagent Preparations

Prepare the following reagents ahead of time before starting your No-Amp experiments:

- CRISPR-Cas9 RNA Oligonucleotides
- PacBio Barcoded Adapters for Multiplexing
- Carrier DNA

CRISPR-Cas9 RNA Oligonucleotides

1. Prepare 50 µM stocks of crRNA and tracrRNA oligos by resuspending the oligos in molecular biology grade nuclease-free water. These oligos are used to create the gRNA in the **Guide RNA Preparation** step as described later in the "CRISPR-Cas9 Digestion" section.

Reagent	To Do	Storage
crRNA 1 (target specific)	Dilute oligo to 50 µM stock	-80°C
crRNA 2 (target specific)	Dilute oligo to 50 µM stock	-80°C
tracrRNA (universal)	Dilute oligo to 50 µM stock	-80°C

Note: The accuracy of the final oligo stock concentration can be improved by targeting a higher initial concentration (10-20% higher) and adjusting down to the final concentration using the measured concentration of the initial resuspension.

2. Aliquot to individual tubes for a maximum of 10 uses per tube to minimize freeze/thaw cycles.
3. Store all 50 µM RNA oligo preparations at -80°C.

PacBio Barcoded Adapters for Multiplexing

1. Resuspend the oligos in molecular biology grade nuclease-free water to 100 µM.
2. Prepare 20 µM stocks of Barcoded Adapters in 1X Annealing Buffer.

Reagent	Stock Conc.		Final Conc.	✓	Notes
Barcoded Adapters	100 µM	10.0 µL	20 µM		
Annealing Buffer*	10X	5.0 µL	1X		
Nuclease-free Water		35.0 µL			
Total Volume		50.0 µL			

* Supplied at 10X concentration in the PacBio No-Amp Accessory Kit.

Note: The accuracy of the final barcoded adapter oligo concentration can be improved by targeting a higher initial working stock concentration (10-20% higher) and adjusting down to the final concentration using the measured concentration of the initial working stock.

3. Anneal the 20 µM working stocks as follows:
   - Incubate in a thermal cycler at 95°C for 5 minutes
   - Ramp to down to 25°C at the maximum cooling rate.
   - Hold at 4°C.
4. Store all adapter preparations at -20°C. Annealed working stocks can be prepared in advance and stored without requiring additional reannealing.

Carrier DNA

Carrier DNA (1 kb DNA Ladder) is required for loading samples prepared with this procedure on the Sequel Systems. It is added to the sample after the polymerase-bound complexes are purified. To prepare the complexes for loading, see the “Purification of Polymerase Bound SMRTbell Complexes” section for more information.

1. Dilute the 1 kb DNA Ladder to a final concentration of 50 ng/µL.

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
Elution Buffer		45.0 µL			
1 kb DNA Ladder	500 ng/µL	5.0 µL	50 ng/µL		

2. Store Carrier DNA at -15 to -25°C.

Dephosphorylation of gDNA Before CRISPR-Cas9 Digestion

Genomic DNA is first dephosphorylated to prevent fragment ends from participating in the ligation reaction following the CRISPR-Cas9 Digestion step. This dephosphorylation step reduces the amount of off-target molecules in the final SMRTbell library.

The required starting gDNA input amount for this procedure is summarized in Table 1.

Prepare the following reaction for each sample. For multiplexing experiments, each sample must be dephosphorylated separately.

Note: The reaction table below was optimized for input gDNA amounts from 500 ng to 5000 ng. For input DNA >5000 ng, scale the volumes 2X. Be sure to maintain the final concentrations of buffer and enzyme. (For example: double the volume of each reaction component when the input gDNA is between 5 µg to 10 µg). If the sample concentration is too low to accommodate the desired input amount, either concentrate the sample or scale-up the reaction.

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
Genomic DNA	____ ng/µL	____ µL for ____ µg	____ ng/µL		
Water (nuclease-free)		____ µL to adjust to Total Volume			
NEBuffer 3.1	10X	8.0 µL	1.2X		
rSAP	1 U/µL	4 µL	0.05 U/µL		
Total Volume		80.0 µL			

2. Mix the reaction thoroughly by gently inverting the microcentrifuge tube at least 20 times.
   Note: To minimize DNA shearing, do not vortex or flick the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 1 hour and then either place on ice or immediately proceed to the next step.
   Note: During the 1-hour incubation, prepare the **Guide RNA** for the Cas9 digestion step.
5. Incubate at 65ºC for 10 minutes to inactivate the phosphatase and then place on ice.
6. Proceed to the next step.

CRISPR-Cas9 Digestion

In this section, target-specific Guide RNAs are prepared first, followed by formation of the guide RNA/Cas9 nuclease complex and then, finally, digestion of the dephosphorylated gDNA.

Guide RNA Preparation

The region of interest requires two target specific-cRNAs (crRNA 1 and crRNA 2). Each cRNA must first be annealed to tracrRNA (universal and not specific to any assay design) separately in a 1:1 volumetric ratio to form gRNAs 1 and 2. The two gRNAs are subsequently pooled in an equimolar mixture.

For experiments targeting multiple regions in the same CRISPR-Cas9 reaction, see **Step 8** below.

The total volume of gRNA prepared below (20.0 µL) is sufficient for Cas9 digestion of two samples with up to 5 µg of input gDNA each. Scale-up the reagent volumes proportionally to accommodate additional samples.

1. To a 0.2 mL PCR tube, prepare gRNA 1.

   Note: Dedicated nuclease-free water and consumables for working with RNA should be used in all steps using RNA oligonucleotides. Keep RNA oligos and associated reagents on ice at all times.

Reagent for gRNA 1	Stock Conc.	Volume	Final Conc.	✓	Notes
crRNA 1	50 µM	1.0 µL	5 µM		
tracrRNA	50 µM	1.0 µL	5 µM		
Nuclease-Free Duplex Buffer		8.0 µL			
Total Volume		10.0 µL			

2. To a separate 0.2 mL PCR tube, prepare gRNA 2:

Reagent for gRNA 2	Stock Conc.	Volume	Final Conc.	✓	Notes
crRNA 2	50 µM	1.0 µL	5 µM		
tracrRNA	50 µM	1.0 µL	5 µM		
Nuclease-Free Duplex Buffer		8.0 µL			
Total Volume		10.0 µL			

3. Mix the reactions well by pipetting or flicking the tubes.
4. Spin the tubes briefly in a mini centrifuge to collect the liquid.
5. In a thermal cycler, incubate the samples at 95°C for 5 minutes followed by 25°C for 5 minutes. End the incubation at 4C.
6. Place the gRNAs on ice after cooling to room temperature.
7. Combine the entire volume of both gRNA 1 and gRNA 2 to form the final 5 µM gRNA mixture. The gRNA mixture is now ready to use in single-target CRISPR-Cas9 digestions. The amount of gRNA prepared according to the above steps is sufficient for performing two digestion reactions.

   Note 1: For multiple-target CRISPR-Cas9 digestions on a sample, combine equal volumes of target-specific gRNA mixtures in a LoBind microcentrifuge tube (example, combine equal volumes of gRNA mixture for target 1, target 2, target 3, etc). A final gRNA concentration of 5 µM must be maintained when combined. Use 8 ul of the final (pooled) gRNA mixture for the digestion reaction. The gRNA mixture is stable for two weeks if stored at -20C.

   Note 2: A large volume of freshly prepared gRNA mixture can be stored at -80°C in small aliquots for future use. Follow Note 1 for storage recommendations after the gRNA aliquot is thawed and used for the first time for a No-Amp experiment.

8. Continue to the next step**.**

Prepare the gRNA and Cas9 Nuclease Complex

The gRNA mixture and Cas9 nuclease are combined to form a gRNA/Cas9 complex. The resulting complex identifies and excises the targeted region from the gDNA.

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed. If the sample to be digested is more than 80 ul, scale up the volume of each reaction component (for example, if the dephosphorylated DNA is 160 µL, double the volume of each reaction component). A master mix can be prepared at this step for digestion of several samples.

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
Water (nuclease-free)		7.0 µL			
NEBuffer 3.1	10X	2.0 µL	1X		
Cas9 Nuclease	20 µM	2.0 µL	400 nM		
gRNA (single or multiple target)	5 µM	8.0* µL	400 nM		
Total Volume		19.0 µL			

*For multiple targets, 8.0 µL contains equal volumes of target-specific gRNA mixtures

2. Mix the reaction well by pipetting.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 10 minutes and then place on ice.

Prepare the Cas9 Digestion

1. Add the following reagents in the order listed. First, add 1 µL of RNase inhibitor to the tube containing the dephosphorylated gDNA sample. Add 19.0 ul of the gRNA/Cas9 nuclease complex (prepared above) to the 81 ul dephosphorylated gDNA and ribonuclease inhibitor.

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
gDNA, dephosphorylated		80.0 µL			
Recombinant ribonuclease inhibitor	40 U/µL	1.0 µL			
Prepared gRNA-Cas9 complex (above)		19.0 µL			
Total Volume		100.0 µL			

2. Mix the reaction thoroughly by gently inverting the microcentrifuge tube at least 20 times.

   Note: To minimize DNA shearing, do not vortex or flick the tube.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 1 hour and then place on ice.
5. Spin the tube briefly in a mini centrifuge to collect the liquid and then return the tube to ice.
6. Immediately proceed to the next step.

AMPure PB Bead Purification of Cas9 Digest Products

Perform one round of purification following the **CRISPR-Cas9 Digestion** step using 0.45X volume of AMPure PB beads. Use a 1.5 mL LoBind microcentrifuge tube for this purification step.

STEP	✓	Instructions	Notes
1		Transfer the Cas9-digested sample to a new 1.5 mL LoBind tube and add enough Elution Buffer to bring the total sample volume to 500 µL.	
2		Add 0.45X volume of AMPure PB beads to the sample. **Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process.	
3		Mix the reaction thoroughly by gently inverting the microcentrifuge tube at least 20 times. Spin the tube briefly to collect the liquid. **Note:** To minimize DNA shearing, do not vortex or flick the tube.	
4		Incubate for 15 minutes on the benchtop to bind the sample to the beads. Do not vortex.	
5		Spin the tube briefly to collect the liquid.	
6		Collect the beads to the side of the tube in a magnetic bead rack. Allow beads to separate for at least 5 minutes or longer until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic bead rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet. **Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. – Do not remove the tube from the magnetic rack. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (1 mL for 1.5 mL tube). – After 30 seconds, slowly remove the 80% ethanol and discard. **Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Accurately measure the volumes ethanol and water added to ensure that the final solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.	
9		Repeat Step 8 above 2 more times for a total of 3 washes.	
10		Remove residual 80% ethanol. – Remove the tube from the magnetic bead rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic bead rack. – Slowly remove any remaining 80% ethanol with a P20 pipette and discard.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat Step 10.	

12		Elute the DNA off the beads. – Add 30 µL Elution Buffer for every 100 µL of Cas9 digestion reaction plus 1 additional µL to the beads (For example: Add 61 µL of Elution Buffer to the beads for a 200 µL Cas9 digestion reaction). Mix the reaction thoroughly by gently inverting several times. – If the beads stick to the tube wall and are not washed down to the bottom with Elution Buffer, let the tube incubate on the bench top for a few minutes and then repeat the gentle mixing step. Repeat the incubation-gentle mixing cycle several times, if necessary, until most of the beads are resuspended in the Elution Buffer and do not appear to be forming a bead pellet. – Incubate for 10 minutes on the benchtop to elute the sample from the beads. Do not vortex. – Spin the tube briefly to collect the liquid and then place the tube back on the magnetic rack. – Let the beads separate fully for at least 5 minutes, and then without disturbing the bead pellet carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice. – Discard the beads.	
13		Verify your DNA amount and concentration using a Qubit fluorometer. – Add 1 µL of eluted sample to 4 µL of Elution Buffer. Use 1 µL of this 5-fold dilution to measure the DNA concentration using the Qubit 1X dsDNA HS Assay Kit according to the manufacturer's recommendations. – Record the concentration and amount of recovered sample. Typical DNA yield is greater than 40% of the starting gDNA input amount. – Proceed to the next step.	

Adapter Ligation

Adapters are blunt-ligated to the purified products of CRISPR-Cas9 cleavage, creating SMRTbell templates with symmetric ends on the target molecules. For a multiplexed library, ligation reactions are pooled during post-ligation sample pooling and cleanup.

Ligation Setup

The reaction table below accommodates up to 30 µL of purified DNA sample. Scale up the volume of each reaction component by the amount listed in the reaction table for every 30 µL increase in input sample volume, maintaining final concentrations listed in the table below (For example: Double the volume of each reaction component when the input sample volume is 60 µL).

Note: Mix where instructed BEFORE adding ligase.

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
gDNA, Cas9-digested		30.0 µL			
Barcoded Adapter, annealed	20 µM	1.0 µL	0.40 µM		
T4 DNA Ligase Reaction Buffer	10X	5.0 µL	1X		
Water (nuclease-free)		12.5 µL			
Mix by gently inverting the tube several times and spin briefly before proceeding					
T4 DNA Ligase	30 U/µL	1.5 µL	0.90 U/µL		
Total Volume		50.0 µL			

2. Mix the reaction thoroughly by gently inverting the microcentrifuge tube at least 20 times.

   Note: To minimize DNA shearing, do not vortex or flick the tube.

3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 16ºC for 2 hours.
5. Inactive the ligase by incubating at 65ºC for 10 minutes and place on ice. Alternatively, a thermal cycler may be used for ligation, followed by heat inactivation and holding at 4ºC overnight.

Sample Pooling

1. Spin the tube at 14,000 RCF for 5 minutes in a microcentrifuge. Note the tube orientation in the centrifuge rotor.
2. Transfer the supernatant to a new LoBind tube taking care not to touch the pipette tip to the outer-facing tube wall where any pelleted material may be located. A pellet may be visible on the sidewall of the tube after the supernatant is removed.
3. Place the new tube with supernatant on ice and discard the tube with pelleted material.
4. If multiplexing, pool samples that are to be combined in an equimolar fashion.
5. Proceed to the next step to concentrate the pooled library.

AMPure PB Bead Purification of SMRTbell Library

Perform one round of purification following the **Adapter Ligation** step using 0.45X volume of AMPure PB beads. Use a 1.5 mL LoBind microcentrifuge tube for this purification step.

STEP	✓	Instructions	Notes
1		Transfer the sample to a new 1.5 mL LoBind tube and add enough Elution Buffer to bring the total sample volume to 500 µL. **Note**: Adding additional Elution Buffer is not necessary when total sample volume is above 500 µL.	
2		Add 0.45X volume of AMPure PB beads to the sample. **Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process.	
3		Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid.	
4		Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous. **Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery.	
5		Spin the tube briefly to collect the liquid.	
6		Collect the beads to the side of the tube in a magnetic rack. Allow beads to separate for at least 5 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet. **Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. – Do not remove the tube from the magnetic rack. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (1 mL for 1.5 mL tube). – After 30 seconds, slowly remove the 80% ethanol and discard. **Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Accurately measure the volumes ethanol and water added to ensure that the final solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.	
9		Repeat Step 8 above 2 more times for a total of 3 washes.	

10		Remove residual 80% ethanol. – Remove the tube from the magnetic rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic rack. – Slowly remove any remaining 80% ethanol with a P20 pipette and discard.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat Step 10.	
12		Elute the DNA off the beads. – Add 100 µL Elution Buffer to the beads for every 5 µg of input gDNA represented by a single sample or pool of samples and mix until homogeneous (For example: Add 200 µL Elution Buffer when input gDNA is between 5 to 10 µg). – Incubate on the bench top for 5 minutes then vortex the tube for 1 minute at 2,000 rpm. – Spin the tube briefly to collect the liquid and then place the tube back on the magnetic rack. – Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice. – Discard the beads. – The purified DNA can be stored overnight at 4ºC or at -20ºC for longer duration before proceeding to the next step.	
13		This is a safe stopping point	

Nuclease Treatment

Failed ligation products and gDNA fragments are removed with a nuclease treatment. Following this, an additional treatment of the SMRTbell library with trypsin facilitates removal of enzymes during the AMPure PB bead purification step.

Digest Setup

The reaction table below accommodates up to 100 µL of purified DNA represented by a single sample or pool of samples. Scale up the volume of each reaction component by the amount listed in the reaction table for every 100 µL increase in input sample volume, maintaining the final concentrations shown in the table below (For example: Double the volume of each reaction component when input sample volume is 200 µL).

1. To a LoBind microcentrifuge tube, add the following reagents in the order listed:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
SMRTbell Library		100.0 µL			
Water (nuclease-free)		67.2 µL			
CutSmart Buffer	10X	20.0 µL	1X		
Exonuclease III	100 U/µL	4.8 µL	1.2 U/µL		
Enzyme A (Enzyme Cleanup Kit)		4.0 µL			
Enzyme B (Enzyme Cleanup Kit)		1.0 µL			
Enzyme C (Enzyme Cleanup Kit)		1.0 µL			
Enzyme D (Enzyme Cleanup Kit)		2.0 µL			
Total Volume		200.0 µL			

2. Mix the reaction well by inverting the microcentrifuge tube at least 20 times.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 2 hours and then place on ice.
5. Immediately proceed to the next step.

Trypsin Treatment

1. To the tube from the previous step, add the following reagents in the order listed:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
SMRTbell Library		200.0 µL			
SOLu-Trypsin	1 mg/mL	9.0 µL	41 µg/mL		
Total Volume		209.0 µL			

2. Mix the reaction well by inverting the microcentrifuge tube at least 20 times.
3. Spin the tube briefly in a mini centrifuge to collect the liquid.
4. Incubate at 37ºC for 20 minutes and then place on ice.
5. Immediately proceed to the next step.

AMPure PB Bead Purification of Nuclease-Treated SMRTbell Library

Perform two rounds of purification following trypsin treatment, using 0.45X volume of AMPure PB beads for the first purification and 0.42X volume of AMPure PB beads for the second purification. Use 1.5 mL LoBind microcentrifuge tubes for both purification steps.

STEP	✓	Instructions for 1st Purification of Nuclease-Treated SMRTbell Library	Notes
1		Transfer the sample to a new 1.5 mL LoBind tube and add enough Elution Buffer to bring the total sample volume to 500 µL. **Note**: Adding additional Elution Buffer is not necessary when total sample volume is above 500 µL.	
2		Add 0.45X volume of AMPure PB beads to the sample. **Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process.	
3		Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid.	
4		Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous. **Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery.	
5		Spin the tube briefly to collect the liquid.	
6		Collect the beads to the side of the tube in a magnetic rack. Allow beads to separate for at least 5 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet. **Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. – Do not remove the tube from the magnetic rack. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (1 mL for 1.5 mL tube). – After 30 seconds, slowly remove the 80% ethanol and discard. **Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Accurately measure the volumes ethanol and water added to ensure that the final solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.	
9		Repeat Step 8 above 2 more times for a total of 3 washes.	

10		Remove residual 80% ethanol. – Remove the tube from the magnetic rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic rack. – Slowly remove any remaining 80% ethanol with a P20 pipette and discard.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat Step 10.	
12		Elute the DNA off the beads. – Add 200 µL Elution Buffer to the beads and mix until homogeneous. – Incubate on the bench top for 5 minutes then vortex the tube for 1 minute at 2,000 rpm. – Spin the tube briefly to collect the liquid and then place the tube back on the magnetic rack. – Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice. – Discard the beads. – Proceed to the second round of AMPure PB bead purification.	

STEP	✓	Instructions for 2nd Purification	Notes
1		Add 0.42X volume of AMPure PB beads to the sample (e.g., add 84 µL of AMPure PB beads for 200 µL of DNA sample recovered from the 1st round of purification). **Note:** Mix the bead reagent well until the solution appears homogeneous before dispensing. Pipette the reagent slowly as the bead mixture is viscous. Precise volumes are critical to the purification process.	
2		Mix the solution thoroughly by vortexing or flicking the tube and then spin briefly to collect the liquid.	
3		Vortex the tube for 10 minutes at 2,000 rpm to bind DNA to the beads. After vortexing, the solution should appear homogeneous. **Note:** We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recovery.	
4		Spin the tube briefly to collect the liquid.	
5		Collect the beads to the side of the tube in a magnetic rack. Allow beads to separate for at least 5 minutes or until the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
6		With the tube still on the magnetic rack, slowly remove the cleared supernatant and save in another tube. Avoid disturbing the bead pellet. **Note:** If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
7		Wash beads with freshly prepared 80% ethanol. – Do not remove the tube from the magnetic rack. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads, taking care not to disturb the bead pellet. Use a sufficient volume of 80% ethanol to fill the tube (400 µL for 0.5 mL tube or 1 mL for 1.5 mL tube). – After 30 seconds, slowly remove the 80% ethanol and discard. **Note:** 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Accurately measure the volumes ethanol and water added to ensure that the final solution is 80% ethanol. 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.	
8		Repeat Step 7 above 2 more times for a total of 3 washes.	
9		Remove residual 80% ethanol. – Remove the tube from the magnetic rack and spin briefly to pellet the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on the magnetic rack. – Slowly remove any remaining 80% ethanol with a P20 pipette and discard.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat Step 9.	
11		Elute the DNA off the beads. – Add 6.3 µL Elution Buffer to the beads and mix until homogeneous. – Incubate on the bench top for 5 minutes then vortex the tube for 1 minute	

		at 2,000 rpm. – Spin the tube briefly to collect the liquid and then place the tube back on the magnetic rack. – Let the beads separate fully and then without disturbing the bead pellet, carefully transfer the supernatant containing DNA to a new LoBind microcentrifuge tube. Keep the purified DNA on ice. – Discard the beads.	
12		– The purified SMRTbell library is now ready for primer annealing and polymerase binding (see next step below). – Alternatively, the SMRTbell library may be stored overnight at 4ºC or at -20ºC before proceeding to the next step. This is a safe stopping point.	

Primer Annealing, Polymerase Binding and Sample Clean-Up

SMRT Link Sample Setup cannot be used with this procedure. Follow the instructions below to perform primer annealing, polymerase binding and sample clean-up.

The primer annealing and polymerase binding instructions below can accommodate a maximum of up 24 µg of input gDNA represented by a single sample or pool of samples.

The primer annealing step is the same for all Sequel Systems. However, the polymerase binding and sample complex purification steps are different for each system. Be sure to follow the instructions specific to the sequencing system used.

Primer Annealing

Sequel System, Sequel II System and Sequel IIe System

1. Prior to setting up the annealing reaction, dilute the Sequencing Primer v4 stock 30-fold by adding 1 µL of primer to 29 µL of Elution Buffer.
2. To a 0.2 mL PCR tube, add the following reagents to condition the sequencing primer before annealing to SMRTbell templates:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
10X Primer Buffer v2	10X	36.0 µL	6.7X		
Diluted Sequencing Primer v4	10X	18.0 µL	3.3X		
Total Volume		54.0 µL			

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin the tube briefly in a mini centrifuge to collect the liquid.
5. Incubate at 80ºC for 2 minutes and then hold at 4ºC or on ice.
6. Transfer the conditioned sequencing primer to a new LoBind microcentrifuge tube and keep on ice until ready to use.

   Note: Any remaining conditioned sequencing primer may be stored at -20°C and used for up to 30 days.
7. To a new 0.2 mL PCR tube, add the following reagents to anneal the conditioned sequencing primer to the SMRTbell templates:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
Conditioned Sequencing Primer v4	3.3X	2.7 µL	1X		
SMRTbell Library		6.3 µL			
Total Volume		9.0 µL			

8. Mix the reaction well by pipetting or flicking the tube.
9. Spin the tube briefly in a mini centrifuge to collect the liquid.
10. Incubate at 20ºC for 1 hour and then hold at 4ºC or on ice.
11. Proceed to the next step.

Polymerase Binding

Sequel System

1. Prepare diluted Sequel DNA Polymerase 3.0 just prior to use by adding 1 µL of polymerase stock to 29 µL of Sequel Binding Buffer and keep on ice. Diluted polymerase must be used immediately. Discard the unused portion.
2. To the PCR tube containing primer-annealed SMRTbell library, add the following reagents in the order listed:

Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
SMRTbell Library with annealed primer from previous step		9.0 µL			
Sequel Binding Buffer	10X	1.5 µL	1X		
DTT	10X	1.5 µL	1X		
Sequel dNTP	10X	1.5 µL	1X		
Mix before proceeding					
Diluted Sequel DNA Polymerase 3.0	10X	1.5 µL	1X		
Total Volume		15.0 µL			

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin the tube briefly in a mini centrifuge to collect the liquid.
5. Incubate at 30ºC for 4 hours and then hold at 4ºC or on ice until ready for sample complex purification.

Sequel II and IIe Systems

1. Dilute the Sequel II DNA Polymerase 2.0 just prior to use by adding 1 µL of polymerase stock to 29 µL of Sequel Binding Buffer and keep on ice. Diluted polymerase must be used immediately. Discard the unused portion.
2. To the PCR tube containing primer-annealed SMRTbell library, add the following reagents in the order listed:

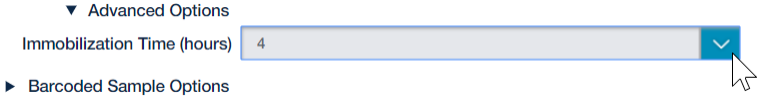
Reagent	Stock Conc.	Volume	Final Conc.	✓	Notes
SMRTbell Library with annealed primer from previous step		9.0 µL			
Sequel Binding Buffer	10X	1.5 µL	1X		
DTT	10X	1.5 µL	1X		
Sequel dNTP	10X	1.5 µL	1X		
Mix before proceeding					
Diluted Sequel II DNA Polymerase 2.0	10X	1.5 µL	1X		
Total Volume		15.0 µL			

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin the tube briefly in a mini centrifuge to collect the liquid.
5. Incubate at 30ºC for 4 hours and then hold at 4ºC or on ice until ready for sample complex purification.

Purification of Polymerase-Bound SMRTbell Complexes

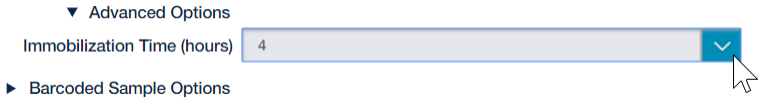
Sequel System

Follow instructions below for purifying complexes for the Sequel System.

STEP	✓	Purification	Notes
1		To a 1.5 mL LoBind microcentrifuge tube, add 35 µL Sequel Complex Dilution Buffer. Then add 15 µL of sample complex.	
2		Add 30 µL AMPure PB beads (0.6X volume) to the tube containing 50 µL diluted sample complex.	
3		Mix well by pipetting or flicking the tube, spin briefly, and incubate for 5 minutes on the bench top to bind sample to the beads. **Note:** Longer incubation times have not been tested and may have a negative impact on polymerase-template complex stability due to high salt concentration.	
4		Collect the beads to the side of the tube in a magnetic rack. Allow the beads to separate for 2 minutes or until the solution appears clear and then slowly remove the supernatant and discard. **Note:** **DO NOT wash the collected bead pellet with ethanol.**	
5		Remove the tube from the magnetic rack and spin briefly to pellet the beads. Place the tube back on the magnetic rack. Slowly remove any remaining supernatant and discard.	
6		Immediately resuspend the beads in 81.0 µL room temperature Sequel Complex Dilution Buffer.	
7		Mix well by pipetting or flicking the tube, spin, and incubate at room temperature for 15 minutes to elute the sample from the beads.	
8		Collect the beads to the side of the tube in a magnetic rack. Allow the beads to separate for 1 minute or until the solution appears clear and then transfer the supernatant to a new LoBind microcentrifuge tube. Keep the tube with sample on ice.	
9		Perform two 100-fold serial dilutions of Sequel DNA Internal Control Complex 3.0 in Sequel Complex Dilution Buffer for a final 10,000-fold dilution. Keep all dilutions on ice.	
10		To the tube containing eluted sample, add 3.0 µL of the 10,000-fold dilution of the DNA internal control complex and 1.0 µL of carrier DNA.	
11		Transfer the sample to a sample plate.	
12		Cover the sample plate and keep at 4ºC or on ice until ready to sequence.	
13		Sequence the sample using an immobilization time of 4 hours, no pre-extension, and a movie collection of time of 20 hours. To specify a custom 4-hour immobilization time, go to the Advanced Options section in SMRT Link Run Design and select 4 hours from the Immobilization Time drop-list menu. 	

Sequel II and IIe Systems

Follow instructions below for purifying complexes for the Sequel II and IIe Systems.

STEP	✓	Purification	Notes
1		To a 1.5 mL LoBind microcentrifuge tube, add 35 µL Sequel Complex Dilution Buffer then add 15 µL of sample complex.	
2		Add 30 µL AMPure PB beads (0.6X volume) to the tube containing 50 µL diluted sample complex.	
3		Mix well by pipetting or flicking the tube, spin, and incubate for 5 minutes on the bench top to bind sample to the beads. **Note:** Longer incubation times have not been tested and may have a negative impact on polymerase-template complex stability due to high salt concentration.	
4		Collect the beads to the side of the tube in a magnetic rack. Allow the beads to separate for 2 minutes or until the solution appears clear and then slowly remove the supernatant and discard. **Note: DO NOT wash the collected bead pellet with ethanol.**	
5		Remove the tube from the magnetic rack and spin briefly to pellet the beads. Place the tube back on the magnetic rack. Slowly remove any remaining supernatant and discard.	
6		Immediately resuspend the beads in 109.6 µL room temperature Sequel Complex Dilution Buffer.	
7		Mix well by pipetting or flicking the tube, spin, and incubate at room temperature for 15 minutes to elute the sample from the beads.	
8		Collect the beads to the side of the tube in a magnetic rack. Allow the beads to separate for 1 minute or until the solution appears clear and then transfer the supernatant to a new LoBind microcentrifuge tube. Keep the tube with sample on ice.	
9		Perform two 100-fold serial dilutions of Sequel II DNA Internal Control Complex 1.0 in Sequel Complex Dilution Buffer for a final 10,000-fold dilution. Keep all dilutions on ice.	
10		To the tube containing eluted sample, add 4.0 µL of the 10,000-fold dilution of DNA internal control complex and 1.4 µL of carrier DNA.	
11		Transfer the sample to a sample plate.	
12		Cover the sample plate and keep at 4ºC or on ice until ready to sequence.	
13		Sequence the sample using an immobilization time of 4 hours, no pre-extension, and a movie collection time of 30 hours. To specify a custom 4-hour immobilization time, go to the Advanced Options section in SMRT Link Run Design and select 4 hours from the Immobilization Time drop-list menu. 	

Appendix A

Recommended guide RNA sequences for targeting *HTT, FMR1, ATXN10, C9orf72* and Ataxia-related repeat elements in the human genome listed in the table below. These custom RNA oligos can be ordered from IDT (or other third-party vendor) and HPLC purification is recommended.

Item	Sequence (5'-3')	Amount Provided
Alt-R® CRISPR-Cas9 tracrRNA (Part Numbers 1072532, 1072533 or 1072534)	universal sequence	5, 20 or 100 nmol scale
Alt-R® CRISPR-Cas9 crRNA, HTT repeat element target, "HTT.DC.1"	CTTATTAACAGCAGAGAACT	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, HTT repeat element target, "HTT.DC.2"	TAAACTTTGAAGACGAGACA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, FMR1 repeat element target, "FMR1.DC.1"	CGCGCGTCTGTCTTTCGACC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, FMR1 repeat element target, "FMR1.DC.2"	CCTTTATGCAAAGTTAGCTC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN10 repeat element target, "ATXN10.DC.1"	TGTTCCACCAGCCTTTGCCA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN10 repeat element target, "ATXN10.DC.2"	TAAATTTCACCTGATCAAGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, C9orf72 repeat element target, "C9orf72.DC.1"	TTGGTATTTAGAAAGGTGGT	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, C9orf72 repeat element target, "C9orf72.DC.2"	GGAAGAAAGAATTGCAATTA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATN1 repeat element target, "ATN1.DC.1"	ACAGGATGCCCAAGGCACTG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATN1 repeat element target, "ATN1.DC.2"	AAAGAGCCCAGTCATGATAG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN1 repeat element target, "ATXN1.DC.1"	TTACGGTGTTCTACACCTCT	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN1 repeat element target, "ATXN1.DC.2"	ATACCTACAGCTGCTACCTG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN2 repeat element target, "ATXN2.DC.1"	TTTGATCACTCAAACTACTC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN2 repeat element target, "ATXN2.DC.2"	TTGACGACCTGCTCCATTGC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN3 repeat element target, "ATXN3.DC.1"	TGTTTTGGCTGTACTTAAAC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN3 repeat element target, "ATXN3.DC.2"	CTATAATATTGATGGCACAG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN7 repeat element target, "ATXN7.DC.1"	AAAAATTGAAAATCTGCATA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN7 repeat element target, "ATXN7.DC.2"	TTAATTTTTTAAGCCCAGGC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN8OS repeat element target, "ATXN8OS.DC.1"	CAATAATCATTAATAGTCAC	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, ATXN8OS repeat element target, "ATXN8OS.DC.2"	ACACTGTGCAATATGTAAGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, BEAN1 repeat element target, "BEAN1.DC.1"	CGAAATCAGTCACTCCCCCA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, BEAN1 repeat element target, "BEAN1.DC.2"	CCATGATCAGGGGTACACTG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, CACNA1A repeat element target, "CACNA1A.DC.1"	GGATGGCTGAAACACTTCGT	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, CACNA1A repeat element target, "CACNA1A.DC.2"	GCACAGCCCCGTTAGCCGGG	2 or 10 nmol scale

Alt-R® CRISPR-Cas9 crRNA, FXN repeat element target, “FXN.DC.1”	CTGCTGTAAACCCATACCGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, FXN repeat element target, “FXN.DC.2”	ACTAAATATGCTGTCCCATG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, NOP56 repeat element target, “NOP56.DC.1”	GACAGCTCCTTTGTAACCAG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, NOP56 repeat element target, “NOP56.DC.2”	AAAGGCTCCAGTATTATGGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, PPP2R2B repeat element target, “PPP2R2B.DC.1”	TATGGTGCTCTGTATAGGGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, PPP2R2B repeat element target, “PPP2R2B.DC.2”	CTTGATTCAGAAAGATACAG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, TBP repeat element target, “TBP.DC.1”	AGGACTGGCTGACTAGTTAG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, TBP repeat element target, “TBP.DC.2”	CTTAACGAATAACTTATACA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, DAB1 repeat element target, “DAB1.DC.1”	AAATATATACACTCTTAGAA	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, DAB1 repeat element target, “DAB1.DC.2”	TAAGTTTACCCATATTCAGT	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, JPH3 repeat element target, “JPH3.DC.1”	CCTTTAAATCCCACTCCCGG	2 or 10 nmol scale
Alt-R® CRISPR-Cas9 crRNA, JPH3 repeat element target, “JPH3.DC.2”	TCAGGGAGTACCCTAAGGGG	2 or 10 nmol scale

Appendix B

Recommended barcoded adapter sequences for the No-Amp application are listed in the table below. These barcoded adapter oligos can be ordered from IDT (or other third-party vendor). Adapters require a 5' phosphorylation modification and HPLC purification is recommended.

The procedure to anneal barcoded adapter oligos to form SMRTbell Adapters is found in the section "PacBio Barcoded Adapters for Multiplexing".

The **Oligo Order Sheet** and **FASTA file** for data analysis are available on our Multiplexing Page.

Item	Sequence (5'-3')
Barcoded Adapter bc1001	/5Phos/CGCACTCTGATATGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACATATCAGAGTGCG
Barcoded Adapter bc1002	/5Phos/CTCACAGTCTGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACACAGACTGTGAG
Barcoded Adapter bc1004	/5Phos/CGCGCGTGTGTGCGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACGCACACACGCGCG
Barcoded Adapter bc1008	/5Phos/CGCAGCGCTCGACTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACAGTCGAGCGCTGCG
Barcoded Adapter bc1009	/5Phos/TCTGTCTCGCGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACACGCGAGACAGA
Barcoded Adapter bc1010	/5Phos/CTCTGAGATAGCGCGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACGCGCTATCTCAGAG
Barcoded Adapter bc1012	/5Phos/ACACGCGATCTAGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACTAGATCGCGTGT
Barcoded Adapter bc1014	/5Phos/ACGCGCGCGTAGTGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTCACTACGCGCGCGT
Barcoded Adapter bc1015	/5Phos/ACACACGTGTCATGCGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCGCATGACACGTGTGT
Barcoded Adapter bc1016	/5Phos/ATACTATCTCTCTATGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCATAGAGAGATAGTAT
Barcoded Adapter bc1017	/5Phos/ATATAGCGCGCGTGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACACGCGCGCTATAT
Barcoded Adapter bc1018	/5Phos/CACAGTGAGCACGTGAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTCACGTGCTCACTGTG
Barcoded Adapter bc1019	/5Phos/ATCTGATAGAGTGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACACTCTATCAGAT
Barcoded Adapter bc1020	/5Phos/ACATCGTCGTGTCGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACGACACGACGATGT
Barcoded Adapter bc1021	/5Phos/ACATCACTATGTATAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTATACATAGTGATGT
Barcoded Adapter bc1022	/5Phos/ATATCACACGTGAGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACTCACGTGTGATAT
Barcoded Adapter bc1023	/5Phos/CAGAGATATCTCTCTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCAGAGAGATATCTCTG
Barcoded Adapter bc1024	/5Phos/CTCTCTGCTCTACATGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCATGTAGAGCAGAGAG

Barcoded Adapter bc1025	/5Phos/GCGCGAGCGTGTCGCGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCGCGACACGCTCGCGC
Barcoded Adapter bc1026	/5Phos/TGTGCGTGTCTCTGTGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCACAGAGACACGCACA
Barcoded Adapter bc1027	/5Phos/TGTGAGAGAGTGTGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTCACACTCTCTCACA
Barcoded Adapter bc1028	/5Phos/GAGAGTCAGAGCAGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTCTGCTCTGACTCTC
Barcoded Adapter bc1029	/5Phos/TCTATAGACATATATAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTATATATGTCTATAGA
Barcoded Adapter bc1030	/5Phos/GAGCGCGATAGAGAGAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTCTCTCTATCGCGCTC
Barcoded Adapter bc1031	/5Phos/CACACACTCAGACATCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGATGTCTGAGTGTGTG
Barcoded Adapter bc1032	/5Phos/CACTATCTCTAGTCTCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGAGACTAGAGATAGTG
Barcoded Adapter bc1033	/5Phos/AGAGACTGCGACGAGAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTCTCGTCGCAGTCTCT
Barcoded Adapter bc1034	/5Phos/ATATCTATATACACATATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATATGTGTATATAGATAT
Barcoded Adapter bc1035	/5Phos/CAGAGAGTGCGCGCGCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGCGCGCGCACTCTCTG
Barcoded Adapter bc1036	/5Phos/GTGTGCGACGTGTCTCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGAGACACGTCGCACAC
Barcoded Adapter bc1037	/5Phos/GTAGTGCGATATGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACATATCGCACTAC
Barcoded Adapter bc1038	/5Phos/GCGCATCGAGACACACATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGTGTGTCTCGATGCGC
Barcoded Adapter bc1039	/5Phos/TGTATCTATGTGTGCGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCGCACACATAGATACA
Barcoded Adapter bc1040	/5Phos/ACACTCTCATATGACAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTGTCATATGAGAGTGT
Barcoded Adapter bc1041	/5Phos/GCGTGCACGCGCGAGAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTCTCGCGCGTGCACGC
Barcoded Adapter bc1042	/5Phos/GCGCTCGTCGAGCGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTCGCTCGACGAGCGC
Barcoded Adapter bc1043	/5Phos/TATGTAGAGCTCTATAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTATAGAGCTCTACATA
Barcoded Adapter bc1044	/5Phos/CGCGCGTCGTCTCAGCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGCTGAGACGACGCGCG
Barcoded Adapter bc1045	/5Phos/AGAGAGTACGATATGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACATATCGTACTCTCT
Barcoded Adapter bc1046	/5Phos/ATATACTCGATATATCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGATATATCGAGTATAT
Barcoded Adapter bc1047	/5Phos/GTGTGTACACATGACAATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATTGTCATGTGTACACAC
Barcoded Adapter bc1048	/5Phos/GAGTGTGAGTGCACACATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGTGTGCACTCACACTC

Barcoded Adapter bc1049	/5Phos/GAGAGAGCACACGTGTATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATACACGTGTGCTCTCTC
Barcoded Adapter bc1050	/5Phos/CTCTCTCGCGTATATCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGATATACGCGAGAGAG
Barcoded Adapter bc1051	/5Phos/GCGCGCGCTAGACACGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCGTGTCTAGCGCGCGC
Barcoded Adapter bc1052	/5Phos/GATATATATCTCACACATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGTGTGAGATATATATC
Barcoded Adapter bc1053	/5Phos/GTGTGACGTACGTGAGATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATCTCACGTACGTCACAC
Barcoded Adapter bc1054	/5Phos/TCTGTAGTGCGTGCGCATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTTGAGAGAGATGCGCACGCACTACAGA

Revision History (Description)	Version	Date
Initial Release.	01	August 2019
Updated recommendation to use Qubit 1X dsDNA HS Assay Kit. Updates made to Reagent Preparation section on page 9, AMPure PB bead Purification on page 15, Adapter Ligation on page 16 (reduced barcoded adapter volume from 10 µL to 1 µL), and Sample Complex Purification on page 25 (increased movie time from 10 hours to 20 hours).	02	September 2019
Added support for Sequel II System using Sequel II Binding Kit 2.0 as reflected in Equipment and Consumables section on page 5, Reagents – PacBio section on page 7, and Sample Complex Preparation section starting on page 24. Updates made to name of ATXN10 RNA oligos in Additional Required Materials section on page 8 and all sample mixing instructions from Post-Digestion section on page 14 through Adapter Ligation section on page 17.	03	September 2019
Incorporation of new methods for improving on-target reads.	04	April 2020
In Digest Setup table, updated final concentration of Exonuclease III from 1.2 U/µL to 2.4 U/µL. In Dephosphorylation Reaction Setup table, changed final concentration of NEBuffer 3.1 from 1.2X to 1X.	05	May 2020
Updated to support 48 plex. Provided additional Guide RNAs and Barcoded Adapters. Procedure is updated to improve flow.	06	Dec 2020